FORM PTO-1390 (REV 10-94) | U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE | ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

12136.1USWO

U S APPLICATION NO (If known, see 37 C F R 1 5)

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US98/25107	01 December 1998 (01.12.98)	02 December 1997 (02.12.97)

TITLE OF INVENTION
NON-TOXIC IMMUNE STIMULATING ENTEROTOXIN COMPOSITIONS

APPLICANT(S) FOR DO/EO/US
BOHACH, Gregory I.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. X This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(l).
4. [ ] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. X A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. has been transmitted by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.

8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).

10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. Below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [X] A FIRST preliminary amendment.
    [ ] A SECOND of SUBSEQUENT preliminary amendment.

14. [ ] A substitute specification.

15. [ ] A change of power of attorney and/or address letter.

16. [X] Other items or information:
    Copy of previously recorded assignment, recorded on 05 December 1997 under Reel/Frame 8997/0584

422 Rec'd PCT/PTO 24 MAY 2000

U.S. APPLICATION NO (If known, see 37 C F R 1.5)X	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/555115	PCT/US98/25107	12136.1USWO

17. x The following fees are submitted:

CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492(a) (1)-(5)): Search Report has been prepared by the EPO or JPO **$840.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.492) **$670.00**	
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) **$690.00**	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$970.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) **$96.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$670.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	**$**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	26 -20 =	6	X **$18.00**	**$108.00**
Independent claims	3 -3 =	0	X **$78.00**	**$**
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$260.00**	**$**
TOTAL OF ABOVE CALCULATIONS =				**$778.00**
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				**$**
SUBTOTAL =				**$778.00**
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f).			+	**$**
TOTAL NATIONAL FEE =				**$778.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property			+	**$**
TOTAL FEES ENCLOSED =				**$778.00**
			Amount to be: refunded	**$**
			charged	**$**

a. X A check in the amount of $778.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. X The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 13-2725. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SIGNATURE:

Ronald A. Daignault
NAME

25,968
REGISTRATION NUMBER

SEND ALL CORRESPONDENCE TO:

MERCHANT & GOULD P.C.
P.O. Box 2903
Minneapolis, MN 55402-0903

S/N Unassigned (Based on PCT/US98/25107) PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	BOHOCH, Gregory	Examiner:	Unassigned
Serial No.:	Unassigned (Based on PCT/US98/25017)	Group Art Unit:	Unassigned
Filed:	Concurrently herewith	Docket No.:	12136.1USWO

(International Filing Date of 01 December 1998)

Title: NON-TOXIC IMMUNE STIMULATING ENTEROTOXIN COMPOSITIONS

PRELIMINARY AMENDMENT

BOX PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

IN THE ABSTRACT

Please insert the attached Abstract page (page 24) into the application as the last page thereof.

IN THE SPECIFICATION

Page 1, after the title, please insert the following paragraph:

--This application is based on International Patent Application PCT/US98/25107 filed December 1, 1998, which is based on U.S. Provisional Application No. 60/067,357 filed on December 2, 1997.—

REMARKS

A new Abstract page is supplied to conform to that appearing on the publication page of the WIPO application, but the new Abstract is typed on a separate page as required by U.S. practice.

Respectfully submitted,

MERCHANT & GOULD P.C.
P.O. Box 2903
Minneapolis, Minnesota 55402-0903
(612) 332-5300

By Ronald A. Daignault
Ronald A. Daignault
Reg. No. 25,968

ABSTRACT

Pyrogenic toxins, such as staphylococcal enterotoxins, modified in the disulfide loop region are provided. The modified toxins retain useful biological properties but have substantially reduced toxicity compared to the corresponding unmodified native toxin. The native pyrogenic toxins are typically modified by deletions within the disulfide loop region to produce modified enterotoxins having 100–fold or greater decrease in toxicity.

NON-TOXIC IMMUNE STIMULATING ENTEROTOXIN COMPOISITIONS

GOVERNMENT SUPPORT

This invention was funded in part by the United States Department of Health under NIH grant R11A128401. The United States Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

A group of biological agents termed superantigens has been described based on their ability to stimulate monocytes and unprimed $CD4^+$ (MHC Class II restricted) and $CD8^+$ (MHC Class I restricted) T-cells. Included with the designation of superantigens are the enterotoxins of *Staphylococcus aureus*.

The enterotoxins of *Staphylococcus aureus* form a group of serologically distinct proteins, originally designated A, B, C_1, C_2, C_3, D, E, G, and H. Subsequently, a number of variants have been described. These proteins and toxic shock syndrome proteins were originally recognized as the causative agents of staphylococcal food poisoning. Ingestion of preformed enterotoxin in contaminated food leads to the rapid development (within two to six hours) of symptoms of vomiting and diarrhea that are characteristic of staphylococcal food poisoning. Toxic shock syndrome toxin-1, TSST-1, a distantly related protein also produced by *S. aureus*, is classically responsible for the toxic shock syndrome, although other staphylococcal enterotoxins may result in the syndrome due to the induction of cytokines.

Other agents which have been identified as superantigens include for example, the staphylococcal exfoliative toxins, A and B; the mammary tumor virus superantigen; rabies virus nucleocapsid protein; pyrogenic exotoxins A, B, C from *S. pyrogens*; and the *Mycoplasma arthritides* mitogen. Additional biological agents which have been demonstrated to have superantigen properties include the human immunodeficiency virus (HIV), gp120 and peptides from HIV, mouse mammary tumor virus and feline immunodeficiency virus. A Leishmania peptide antigen has also been disclosed as a superantigen.

Superantigens, unlike conventional antigens, do not require processing *in-vivo*. In general, superantigens have two binding regions, one of which interacts with the Class II major histocompatibility complex (MHC) on the

antigen presenting cell and the other which interacts with the Vβ variable region of the T−cell receptor on CD4 and/or CD8 cells. Various enterotoxins bind to one or more of the different Vβ receptor epitopes. In contrast to conventional antigens, superantigens do not occupy the T−cells receptor cleft but are felt to bind to an external region thus explaining the ability to activate a broad population of T−cells.

Enterotoxins produced by *Staphylococcus aureus* include a group of related proteins of about 20 to 30 Kd. The complete amino acid composition of a number of staphylococcal enterotoxins and streptococcal pyrogenic exotoxin has been reported (see e.g., PCT Patent Appl. No. WO 93/24136.)

Staphylococcal enterotoxins ("SEs") were initially classified on the basis of their antigenic properties into groups A, B, C1, C2, C3, D, and E. Subsequent relatedness was based on peptide and DNA sequence data. Among the staphylococcal enterotoxins, groups B and C are closely related and groups A, D, and E are closely related in amino acid sequence. SEC1, SEC2, and SEC3 and related isolates share approximately 95% sequence similarity. Table 1 shows the alignment of the predicted sequences of the eight known SEC variants following cleavage of the signal peptide. The N−terminus of each of the mature proteins was verified by amino acid sequencing. Amino acid positions that contain residues that are not conserved among all SEC are indicated by asterisks. SEB and SEC are approximately 45−50% homologous. In contrast, non−enterotoxin superantigens, TSST−1 and *Streptococcal Pyrogenic Enterotoxin C* (SPEC) share only approximately 20% primary sequence homology to SEC. Despite these differences, the tertiary structure of the various enterotoxins show nearly identical folds.

The staphylococcal enterotoxins A, B, C_1, C_2, C_3, D, E, G and H share a common structural feature of a disulfide bond not present in other enterotoxins. Table 2 shows the position of the disulfide bond in a number of enterotoxins. Data in reference to the active sites of the enterotoxin molecule in relationship to biological activity, MHC binding, and TCR binding has been obtained. Sequence data demonstrate a high degree of similarity in four regions of the enterotoxins (See Table 3). The peptides implicated in potential receptor binding correspond to regions 1 and 3 which form a groove in the molecule. Amino acid residues within and adjacent to the α_3 cavity of SEC3 have been shown to relate to T−cell activation.

Table 2.

LOCATION OF DISULFIDE LOOP IN STAPHYLOCOCCUS ENTEROTOXINS

ENTEROTOXIN	AMINO ACID RESIDUES	AMINO ACID SEQUENCE OF DISULFIDE LOOP
SEA	96–106	96→CAGGTPNKTAC
SEB	93–114	93→CYFSKKTNDINSHQTPKRKTC
SEC1	93–110	93→CYFSSKDNVGKVTGGKTC
SEC2	93–110	93→CYFSSKDNVGKVTGGKTC
SEC3 FRI 913	93–110	93→CYFSSKDNVGKVTGGKTC
SEC3 FRI 909	93–110	93→CYFSSKDNVGKVTSGKTC
SEC 4446	93–110	93→CYFSSKDNVGKVTGGKTC
SEC–Bovine	93–110	93→CYFSSKDNVGKVTGGKTC
SEC–Ovine	93–110	93→CCFSSKDNVGKVTGGKTC

The staphylococcal enterotoxins are potent activators of T–cells, resulting in proliferation and the generation of cytotoxic T–cells. SEA is a potent T–cell mitogen eliciting strong polyclonal activation at concentrations of 10^{-13} to 10^{-10} molar in human systems.

The staphylococcal enterotoxins, aside from the acute gastroenteritis and toxic shock syndrome associated with them, have been shown to have a variety of other beneficial biological effects. The biological effects of these agents and the toxic shock syndrome are due in part to the ability of staphylococcal enterotoxins to induce cytokines. Various cytokines described include IL–1, IL–2, and tumor necrosis factor ("TNF"). More recently SEB and toxic shock syndrome toxin ("TSST–1") have been shown to induce interleukin–12, an inducer of cell mediated immunity, in human peripheral blood mononuclear cells. (See Leung et al., *J Exp Med*, 181:747 (1995)). The antitumor activity of treating cancer in rabbits utilizing 40 to 60 μg/kg of a staphylococcal enterotoxin has been disclosed in PCT Patent Appl. Nos. WO 91/10680 and WO 93/24136.

Exposure to enterotoxin either *in–vitro* or *in–vivo* leads to depletion of T–cells having the appropriate Vβ receptor through programmed cell death in some strains of mice, specifically Balb/c and CBA/2. Cell death can be prevented by

high doses of retinol or RU−38486. Programmed cell death has not been observed upon exposure of human cells to enterotoxins.

Although the systemic lethal toxicity of enterotoxins has been related to their ability to induce cytokines, particularly IL−1, IL−2 and gamma interferon, lethal toxicity also appears to be related to a synergistic activity with endogenous endotoxins and the ability of the liver to detoxify endotoxins. Although a number of animals have been utilized to evaluate lethality, the accepted model is the continous infusion over a period of time, usually 4 days, in rabbits. The direct toxic dose varies among various species. The 50% lethal dose of TSST−1 is approximately 50 μg/kg for Balb/c mice. Piglets, although showing clinical manifestations of toxic shock syndrome, tolerate doses of 100 μg/kg of TSST−1. TSST−ovine is known to be non−toxic at doses of 200 μg in rabbits.

In Dutch belted rabbits, intramuscular injection of 50 mg/kg of staphylococcal enterotoxin B caused death. Intravenous injection at 0.5 to 1.0 mg/kg of enterotoxin A or B in rhesus macaques results in hypotension and death (Liu C.T., et al *Amer J Vet Res* 39:279 and 1213, 1978).

In contrast to other species, man is extremely sensitive to enterotoxins. One (1) mg of TSST−1, approximately 15 nanogram/kg, can be lethal for man. Therefore, the recommended doses currently proposed in the art for treating man are unacceptable. There is a need, therefore, for mutant staphylococcal enterotoxins which are non−toxic at anticipated doses for man while still retaining desirable biological activity.

Several studies of staphylococcal enterotoxin have identified a number of biologically active modified or mutant enterotoxins with reduced toxicity. Carboxymethylation of SEB results in a loss of gastrointestinal toxicity but not mitogenic activity. Studies with the TSST−1 have demonstrated the active site to be between amino acids residue 115 and 141. Point mutation of site 135 from histidine to alanine results in a loss of mitogenic activity and toxicity (See Bonventre P.F., et al. *Infect Immun* 63:509 (1995)). Studies with the staphylococcal enterotoxin SEC1 demonstrated that the disulfide bond between residue 93 and 110 is not required for activity (See Hovde et al., *Mol Microbiol* 13:897 (1994)). Studies of the molecular binding region of staphylococcal enterotoxin B using overlapping peptides demonstrated peptide 124 to 154 inhibited SEB induced mitogenic activity.

Based on the known biological activities of the toxic native enterotoxins, it is desirable to create mutants which are at least 1000−fold or more

less toxic compared to native enterotoxins and retain biological activity. Recent studies have demonstrated that mutant enterotoxins can be produced which retain certain biological activities and which may be significantly less lethal as determined in rabbits. A mutant of the TSST-1 enterotoxin which differs in amino acid 136 and is non-lethal at ten times the lethal dose of the native toxin (in rabbits), but retains biological activity has been disclosed. A number of mutants of SEC1, unable to form a disulfide bond, have been reported to be ten times less toxic than the native toxin while retaining biological activity. (See e.g., Hovde et al., *Molec Microbiol* 13:897 (1994)).

SUMMARY OF THE INVENTION

The present invention relates to modified versions of disulfide loop-containing bacterial pyrogenic toxins. The modified pyrogenic toxins retain useful biological properties but have substantially reduced toxicity (e.g., toxicity reduced by at least about 10-fold) compared to the corresponding unmodified native toxin. Selected deletions within the disulfide loop region can produce modified toxins having a 100-fold or greater decrease in toxicity. The toxicity of the modified toxin can be measured based on a variety of parameters, including emetic response inducing activity, fever inducing activity, and lethality (as measured by LD_{50} in Dutch Belted rabbits).

Examples of the present modified toxins include disulfide loop region deletion mutants of native toxins derived from *Staphylococcus aureus* or *Streptococcus pyrogenes*. Suitable native disulfide loop-containing toxins which may be modified according to the present invention include Type A, B, C, D and E staphylococcal enterotoxins as well as streptococcal pyrogenic enterotoxin A ("SPEA") and streptococcal superantigen ("SSA") produced by *S. pyrogenes*.

The pyrogenic toxins constitute a family of exotoxins produced by species of gram positive cocci, such as Staphylococcus and Streptococcus. The pyrogenic toxins are characterized by shared ability to induce fever, enhance host susceptibility to endotoxin shock, and induce T cell proliferation through action as superantigens. Examples of pyrogenic toxins include TSST-1, staphylococcal enterotoxins (SEs), and streptococcal pyrogenic exotoxins (SPEs). In addition to the activities listed above, some pyrogenic toxins have additional activities that are not shared by all pyrogenic toxins. For example, the staphylococcal enterotoxins induce emesis and diarrhea when ingested. Structurally, the pyrogenic toxins have varying

degrees of relatedness at the amino acid and nucleotide sequence levels. A number of the pyrogenic toxins include a disulfide loop as a structural feature. The staphylococcal enterotoxins have a disulfide loop, as do some others in this family. Examples of other pyrogenic toxins that have a disulfide loop are the streptococcal superantigen ("SSA") and streptococcal pyrogenic exotoxin A ("SPEA").

The pyrogenic toxins have varying degrees of relatedness which provides the basis for separating some of them informally into subgroups. One subgroup includes staphylococcal type B and C enterotoxins ("SEB" and "SEC"), as well as SPEA and SSA. These toxins share between about 49% to greater than 95% amino acid sequence homology (Reda et al, Infect. Immun., 62:1867–1874: (1994)). Another subgroup of related pyrogenic toxins include staphylococcal type A and E enterotoxins (SEA and SEE) which are 83% homologous to each other (Couch et al, J. Bacteriol., 70:2954–2060 (19___), less so but significantly to SED (Bayles et al., J. Bacteriol., 171:4799–4806 (1989)). The amino acid sequences of this second subgroup is more distantly related to SEB, SEC, SPEA, and SSA. Examples of pyrogenic toxins having disulfide bonds are present in both of these two subgroups. TSST–1 and streptococcal pyrogenic exotoxins B and C (SPEB and SPEC) are examples of a third subgroup of less related toxins. Although toxins from this third subgroup may share some conserved regions (see table 3) with toxins from the other subgroups, there is little overall sequence homology between toxins in the third subgroup and the pyrogenic toxins in the other two subgroups. Neither TSST–1, SPEB nor SPEC includes a disulfide loop.

The disulfide loop region of a native pyrogenic toxin, such as a native staphylococcal enterotoxin, is generally modified through deletion of a number of amino acid residues within the loop. The modification typically includes deletion of amino acid residues within the disulfide loop region and may include one or more substitutions and/or additions to the remaining loop residues. After modification, the disulfide loop region typically contains no more than about 10 and, preferably, no more than about 6 amino acids residues. In another embodiment of the invention, a modified pyrogenic toxin is formed from a native pyrogenic toxin modified by deletion of at least 40% of the amino acid residues within the disulfide loop region, e.g., by deletion of 8 or more amino acid residues from the disulfide loop region of a native type C staphylococcal enterotoxin.

The present invention is also directed to isolated nucleic acids which include a nucleotide sequence encoding a modified pyrogenic toxin.

DETAILED DESCRIPTION OF THE INVENTION

The current invention relates to modified versions of pyrogenic toxins, such as staphylococcal enterotoxins produced by modifications which include deletions in the disulfide bond region. The present invention describes the feasibility of obtaining mutant pyrogenic toxins which retain biological activity but demonstrate significantly lower toxicity at doses well in excess of the normal lethal dose and levels anticipated for human therapeutic use.

The modified pyrogenic toxins are derived from a native pyrogenic toxin having a disulfide loop. The terms "disulfide loop" and "disulfide loop region" are used interchangeably herein. As employed in this application, these terms refer to the sequence of about 10 to about 30 amino acid residues forming a loop defined by a disulfide bond in a native pyrogenic toxin. The term "disulfide loop region" also refers to the corresponding portion of the sequence of a modified pyrogenic toxin which has been produced by deletion, substitution or addition of one or more amino acid residues of the disulfide loop of a native pyrogenic toxin. The disulfide loop region is defined to begin with the N–terminal Cys residue and end with the C–terminal Cys residue of the loop, e.g., amino acid residues 93–110 of staphylococcal enterotoxin C1. As used herein, the positions of the disulfide loop region for a given native pyrogenic toxin are numbered beginning with the N–terminal cyteine residue in the loop, e.g., position 93 of type B or C staphylococcal enterotoxins is also referred to herein as position 1 of the disulfide loop region.

The modification of the disulfide loop typically includes deletion of at least about 40% of the amino acid residues within the disulfide loop. For example, this generally results in the deletion of at least about 8 amino acid residues from the disulfide loop region of an SEC. Examples of native staphylococcal enterotoxin which can be modified to form the present low toxicity toxins include type A, B, C, D, E, G, and H staphylococcal enterotoxins. Type C staphylococcal enterotoxins such as staphylococcal enterotoxin C1, staphylococcal enterotoxin C2, staphylococcal enterotoxin C2, staphylococcal enterotoxin C–MNCopeland, staphylococcal enterotoxin C–4446, staphylococcal enterotoxin C–bovine (GenBank Accession No. L13374), staphylococcal enterotoxin C–canine (GenBank Accession No. V19526) and staphylococcal enterotoxin C–ovine (GenBank Accession No. L13379) are particularly suitable enterotoxins for modification by deletion of a portion of the disulfide loop region to form a staphylococcal enterotoxin with decreased toxicity.

The modified disulfide loop region generally contains no more than about 10 amino acid residues and, preferably no more than 6 amino acid residues. For example, a type C staphylococcal enterotoxin, such as staphylococcal enterotoxin C1, can be modified to delete amino acid residues 98–106 (residues 6–14 of the disulfide loop) to form a modified staphylococcal enterotoxin having substantially reduced toxicity. An even greater reduction in toxicity is produced by deleting amino acid residues 95–106 (disulfide loop residues 3–14) of staphylococcal enterotoxin C1. Both of these mutants, despite having substantially reduced toxicity, are biologically active as evidenced by their ability to stimulate the uptake of thymidine by human peripheral blood mononuclear cells.

In addition to deletion of a number of the disulfide loop residues, substitution of a cysteine residue for the residue at position 2 of disulfide loop can contribute to a decrease in the toxicity of a pyrogenic toxin such as a type C staphylococcal enterotoxin. In a preferred embodiment, staphylococcal enterotoxin C1 can be modified so that in addition to deletion of a substantial portion of the residues in the loop region, the amino acid residue at position 2 of the loop, Tyr–94, is replaced by a cysteine.

Other examples of preferred embodiments of the invention include modified staphylococcal enterotoxins having no more than 10 amino acid residues and, preferably, no more than 6 amino acid residues in the disulfide loop region. Particularly preferred examples include staphylococcal enterotoxins with no more than about 9 amino acid residues which include the sequence Cys–Gly–Lys–Thr. One example of such a mutant is staphylococcal enterotoxin C1 modified to have the disulfide loop region sequence Cys–Cys–Gly–Lys–Thr–Cys.

The methods of this invention employed in preparing mutant enterotoxins, and their screening, analysis, and purification are known in the art and described herein. Site directed mutagenesis can be carried out by initially cloning the SEC1 structural gene SEC_{MNDON} on a 1.4 Kb MindIII–BamH1 restriction fragment. A unique *SphI* restriction site (5'–GCATGC–3') can be introduced into SEC_{MNDON} in the region coding for the disulfide loop at nucleotides 301–306 (5'–GTAGGT–3') using a commercially available kit (Altered Sites *in vitro* Mutagenesis System, Promega). Potential mutants may be screened by *SphI* digestion and confirmed by nucleic acid sequence analysis.

Select SEC_{MNDON} deletion mutants were cloned into an *E. coli* cell line using pMIN164 an *E. coli* – *S. aureus* shuttle vector. To facilitate protein

purification, recombinant plasmids from *E. coli* RR1 transformants were transformed into *S. aureus* RN4220 by standard protoplast transformation techniques. Plasmids containing cloned toxin genes were maintained in *S. aureus* RN4220 under erythromycin (50 mg/ml) selection. For purification of native and mutant derivatives of the toxin recombinants, *S. aureus* RN4220 cultures were grown in dialyzable beef heart media supplemented with 1% glucose buffer (330 mM glucose; 475 mM $NaHCO_3$; 680 mM NaCl; 137 mM $Na_2HPO_4.H_2O$; 28 mM L-glutamine) and erythromycin (50 mg/ml) followed by ethanol precipitation.

Purification of ethanol precipitated proteins was accomplished by preparative flat bed isoelectric focusing (IEF). Following the IEF run, proteins in select fractions were pooled, dialyzed to remove the amphylotes and then visually assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The toxicity and biological activity of the mutants was evaluated using standard methods known to those skilled in the art as described below. The emetic activity of SEC1, and the SEC1 mutant toxins was determined by a modification of the standard monkey feeding assay using young adult pigtail monkeys (*M. nemestrina*). The animals were manually restrained while the toxin was administered through a nasogastric tube (Infant feeding tube; Becton Dickinson, Rutherford, NJ). Toxins were screened for retention of emetic activity at a dose of 10 µg/Kg which is approximately 100 times the minimal emetic dose for SEC1. Non-emetic toxins were tested for residual emetic activity at a high dose of 250 µg/Kg.

The mitogenic capacities of mutant toxins were compared to that of SEC1 native toxin by using human peripheral blood mononuclear cells (PMBC) in a standard 4-day assay. Solutions of native and mutant toxin were added in triplicate to PBMC cell suspensions, $1x10^6$ cells/ml, in 96-well tissue culture plates. This mixture of cells and toxin was then incubated at 37°C under atmospheric conditions of 6% CO_2 for 72 hours. [^{3}H]-thymidine (New Research Products, Boston, MA) at a concentration of 1 µCi/25 µl in a complete RPMI medium was added to each well and allowed to incubate under the same conditions for an additional 18-24 hours. After incubation, radiolabeled cellular nucleic acids were harvested onto glass fiber filters (Skatron, Sterling, VA) using a semi-automatic cell harvester (Skatron). Lymphocyte proliferation was quantitated by measuring incorporation of [^{3}H]-

thymidine into cellular DNA using a liquid scintillation counter (TRI–CARB 1500 Liquid Scintillation Center, Packard, Rockville, MD).

The ability of a staphylococcal enterotoxin to induce a fever response and enhance susceptibility of lethal endotoxic shock can be determined *in vivo* using a standard rabbit model (Bohach et al., Infect. Immun., 55, 428 (1987)). Following conditioning in a test rack and having baseline body temperature recorded, adult New Zealand White rabbits can be initially intravenously injected with a native or mutant toxin at a concentration of 10 μg/kg in sterile physiological saline. Sterile saline and purified SEC1 toxin are typically used as negative and positive controls respectively. Following toxin injection, rabbit body temperature is generally monitored rectally every hour for four hours. Four hours after initial treatment, an intravenous injection of lipopolysaccharide ("LPS") from *Salmonella typhimurium* (Difco Laboratories, Detroit, Michigan) is administered intravenously at a concentration of 10 μg/Kg in sterile saline. Animals are then observed for signs of shock and mortality for 48 hours after LPS injection.

Cytokine induction may be determined by utilizing isolated mononuclear cells from heparinized venous blood. Briefly, heparinized venous blood is obtained and layered onto lymphocyte separation medium. The tubes are spun and the mononuclear layer is harvested, washed in PBS, resuspended in RPMI containing 10% FCS and adjusted to 1×10^6 cells per ml. Aliquots of 100 μl are typically placed into 96–well microtiter plates. The enterotoxins were added in 100 μl to a final concentration of 1 ng/ml. Following incubation at 37°C for 48–72 hours, the supernatant was harvested can be assayed for cytokines using commercially available kits from R&D Systems Minneapolis, MN.

The invention will be further described by reference to the following examples. These examples illustrate but do not limit the scope of the invention that has been set forth herein. Variation within the concepts of the invention will be apparent.

EXAMPLE 1

The SEC1 structural gene, SEC_{MNDON}, was previously cloned on a 1.4 Kb *HindIII–BamH1* restriction fragment (Bohach et al., Infect. Immun., 55, 428 (1987)). A unique *SphI* restriction site (5'–GCATGC–3') was introduced into SEC_{MNDON} in the region coding for the disulfide loop at nucleotides 301–306 (5'–GTAGGT–3') using a commercially available kit (Altered Sites *in vitro* Mutagenesis

System, Promega). Potential mutants were screened by *Sph1* digestion and confirmed by nucleic acid sequence analysis.

EXAMPLE 2

The unique *Sph1* site was used to linearize the SEC_{MNDON} gene so that bi-directional deletions could be generated using *Bal-31* exonuclease (Boehringer Mannheim, Indianapolis, IN). *Bal-31* generated deletion mutant plasmids were ligated and transformed into *E. coli* TG1. Transformants growing on LB-AMP (125 μg/ml) with in-frame stable deletions were detected by screening with SEC1 specific rabbit antisera in immunodiffusion and analyzed by nucleic acid sequence analysis. Sequencing reactions were done using *Sequenase Version 2.0*, a commercially available kit (U.S. Biochemical Corp., Cleveland, Ohio).

Three deletion mutants were chosen for detailed analysis based on the size and location of the deletions in the loop region of the toxin; SEC1-4AA, SCE1-9AA, and SEC1-12AA. Following purification, each of the disulfide loop mutant toxins could be distinguished from wild type SEC1 on the basis of size when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The nucleic acid and amino acid sequences for SEC1 and three deletion mutants are shown in Table 4.

EXAMPLE 3

Mutant Toxin Purification

Select SEC_{MNDON} deletion mutants were cloned into *E. coli* RR1 using pMIN164, an *E. coli – S. aureus* shuttle vector. To facilitate protein purification, recombinant plasmids from *E. coli* RR1 transformants were transformed into *S. aureus* RN4220 by standard protoplast transformation techniques. Plasmids containing cloned toxin genes were maintained in *S. aureus* RN4220 under erythromycin (50 μg/ml) selection. For purification of the recombinantly produced native and mutant derivatives of the toxin, *S. aureus* RN4220 cultures were grown in dialyzable beef heart media supplemented with 1% glucose buffer (330 mM glucose; 475 mM $NaHCO_3$; 680 mM NaCl; 137 mM $Na_2HPO_4.H_2O$; 28 mM L-glutamine) and erythromycin (50 μg/ml) followed by ethanol precipitation.

Purification of ethanol precipitated proteins was accomplished by preparative flat bed isoelectric focusing ("IEF"). Following the IEF run, proteins in

select fractions were pooled, dialyzed to remove the ampholytes and then visually assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

EXAMPLE 4

Emesis Assay

Assays of the emetic activity of SEC1 and the modified SEC1 toxins were conducted using a modification of the standard monkey feeding assay using young adult pigtail monkeys (*M. nemestrina*). The animals were manually restrained while the toxin was administered through a nasogastric tube (Infant feeding tube; Becton Dickinson, Rutherford, NJ). Toxins were screened for retention of emetic activity at a dose of 10 μg/Kg which is approximately 100 times the minimal emetic dose for SEC1. Non–emetic toxins were tested for residual emetic activity at a high dose of 250 mg/Kg.

It has been previously shown that the minimal emetic dose of wild type SEC1 for *M. nemestrina* was 0.1 μg/Kg. For initial experiments in which the emetic ability was tested, loop mutant toxins were administered at 10 μg/Kg. This insured an excess of toxin over the wild type SEC1 minimal emetic dose. Following intragastric toxin inhibition, animals were observed for at least 12 hours for an emesis response. SEC1–12AA did not show emesis at the 10 μg/Kg concentration and was subsequently tested for emesis at a higher toxin concentration, 250 μg/Kg. Even at this higher doseage level, the SEC1–12AA loop mutant toxin showed no emetic response (See Table 5).

Table 5.

Emetic Response[c] of SEC1 Loop Deletion Mutants

Dose[a]	SEC1	SEC1−4aa	SEC1−9aa	SEC1−12aa
250 μg/Kg	ND[b]	ND	ND	0/2
10 μg/Kg	2/2	2/2	2/2	0/2
1 μg/Kg	2/2	2/2	0/1	ND

[a] μg Toxin/Kg Body Weight
[b] ND = Not Determined
[c] Number animals exhibiting emetic response/Total number of animals

EXAMPLE 5

Pyrogenicity and Enhancement of Lethal Endotoxic Shock

The SEC1 mutant toxin's ability to induce a fever response and enhance susceptibility of lethal endotoxic shock was determined *in vivo* using a previously described rabbit model. Following 1 hour of conditioning in a test rack and having baseline body temperature recorded, adult New Zealand White rabbits were initially intravenously injected with SEC1 or SEC1 mutant toxin at a concentration of 10 μg/Kg in sterile physiological saline. Sterile saline and purified SEC1 toxin were used as negative and positive controls, respectively. Following toxin injection, rabbit body temperature was monitored rectally every hour for four hours. Four hours after initial treatment an intravenous injection of lipopolysaccharide (LPS) from *Salmonella typhimurium* (Difco Laboratories, Detroit, Michigan) was administered intravenously at a concentration of 10 μg/Kg in sterile saline. Animals were watched for signs of shock and mortality for 48 hours after LPS injection.

Wild type SEC1, administered at both 10 μg/Kg and 1 μg/Kg, showed a typical temperature rise (Table 6) as well as an enhanced susceptibility to endotoxic shock (Table 7).

Table 6.

Maximum Temperature Rise in °C in Rabbits Given Noted Amount of Native SEC1, Compound to SEC1–4, SEC1–9, or SEC1–12 Mutant

	SEC1	SEC1–4aa	SEC1–9aa	SEC1–12aa
100 µg	ND	ND	ND	0.6
10 µg	1.6	1.46	0.9	0.45
1 µg	1.3	1	0.43	ND
0.1 µg	1.05	0.65	ND	ND
0.01 µg	0.475	0.425	ND	ND

Table 7.

Lethality in Dutch Belted Rabbits of Native SEC1 and SEC1 Deletion Mutants

Dose (µg)	SEC1	SEC1–4	SEC1–9	SEC1–12
100	ND	ND	ND	0/3
10	3/3	3/3	2/3	0/3
1	2/2	3/3	0/3	ND
0.1	3/3	2/3	ND	ND
0.01	1/4	0/3	ND	ND

SEC1–12AA loop mutant toxin concentration was increased to 100 µg/Kg after test animals showed both reduced pyrogenic effects and no susceptibility to endotoxin shock at the initial dose tested, 10 µg/Kg. Following this log fold increase of the SEC–12AA mutant toxin to 100 µg/Kg it was seen that the mutant toxin induced a slight temperature increase over the initial SEC1–12AA loop mutant toxin concentration tested but was not lethal in the assay.

EXAMPLE 6

Mitogenicity

The mitogenic capacity of mutant toxin was compared to that of SEC1 native toxin by using human peripheral blood mononuclear cells (PMBC) in a standard 4–day assay (Bohach et al., Infect. Immun., 55, 428 (1987)). Human peripheral blood mononuclear cells were isolated from 30 to 60 ml of heparinized blood obtained by venipuncture. The blood was mixed 1:1 with Dulbecco's Phosphate Buffered Saline (PBS) (without Calcium or magnesium) and layered onto Fico/Lite (density 1.079 g/ml, Atlanta Biologicals, Norcross, GA) and centrifuged at 400 x g for 20 minutes. The interface containing the mononuclear cells was removed and washed 3 times with PBS. After the last wash, the pellet was resuspended in Hank's Balanced Salt solution without calcium or magnesium and layered on Fetal Bovine Serum (Atlanta Biologicals, Norcross, GA) and centrifuged at 100 x g for 10 minutes to remove the platelets. The cells were washed in Hank's Balanced Salt solution, Cat. No. M1211–021–LV (Atasca, IL). A cell count was done by Trypan Blue exclusion.

Solutions of native and mutant toxins were added in triplicate to PMBC cell suspensions, $1x10^6$ cells/ml, in 96 well tissue culture plates. This mixture of cells and toxin was then incubated at 37°C under atmospheric conditions at 6% CO_2 for 72 hours. [^{3}H]–thymidine (New Research Products, Boston, MA) at a concentration of 1 μCi/25 μl in a complete RPMI medium was added to each well and allowed to incubate under the same conditions for an additional 18–24 hours. After incubation, radiolabeled cells were harvested onto glass fiber filters (Skatron, Sterling, VA) using a semi–automatic cell harvester (Skatron). Lymphocyte proliferation was quantitated by measuring incorporation of [^{3}H]–thymidine into cellular DNA using a liquid scintillation counter (TI–CARB 1500 Liquid Scintillation Counter, Packard, Rockville, MD).

Using SEC1 wild type toxin as a control it was determined that the SEC1–12AA loop mutant toxin showed mitogenic activity at a reduced effectiveness compared to native SEC1 and the SEC1–4 and SEC1–9 mutants in stimulating PMBC cells (see Table 8).

Table 8.

SEC1 Loop Mutant Mitogeneicity [^{3}H]−Thymidine Uptake (in CPM) of Enterotoxin Stimulated Human Peripheral Blood Mononuclear Cels

Dose	SEC1	SEC1−4AA	SEC1−9AA	SEC1−12AA
0.1 pg	1918	2067	1067	978
1 pg	8533	7453	2360	954
0.01 ng	14138	15495	7682	1200
0.1 ng	21557	23689	21481	6500
1 ng	30329	28892	28951	9400
0.01 µg	33089	31640	23333	2700

All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains and are hereby incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

Table 1.
Amino Acid Sequence of Selected Staphylococcal Enterotoxins

```
                                                                         40
                       *   *    * *   * *   *                            ↓*                 *  *  *                   *  *
SEC1            1 ESQPDPTPDELHKASKFTGLMENMKVLYDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEGLAKKYKDE   80
SEC2            1 ESQPDPTPDELHKSSEFTGTMGNMKYLYDDHYVSATKVMSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE   80
SEC3-FRI913     1 ESQPDPMPDDLHKSSEFTGTMGNMKYLYDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE   80
SEC3-FRI909     1 ESQPDPMPDDLHKSSEFTGTMGNMKYLYDDHYVSATKVKSVDKFLAHDLIYNINDKKLNNYDKVKTELLNEDLANKYKDE   80
SEC-MNCopeland  1 ESQPDPTPDELHKSSEFTGTMGNMKYLYDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE   80
SEC-4446        1 ESQPDPMPDDLHKSSEFTGTMGNMKYLYDDHYVSATKVKSVDKFLAHDLIYNISDKRLKNYDKVKTELLNEDLAKKYKDE   80
SEC-bovine      1 ESQPDPTPDELHKASKFTGLMENMKVLYDDRYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE   80
SEC-ovine       1 ESQPDPTPDELHKASKFTGLMENMKVLYDDRYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE   80
                                                                          120
                              *              *                          ↓         *       *
SEC1           81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD  160
SEC2           81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD  160
SEC3-FRI913    81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLVRVYENKRNTISFEVQTDKKSVTAQELD  160
SEC3-FRI909    81 VVDVYGSNYYVNCYFSSKDNVGKVTSGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD  160
SEC-MNCopeland 81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD  160
SEC-4446       81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLVRVYENKRNTISFEVQTDKKSVTAQELD  160
SEC-bovine     81 VVDVYGSNYYVNCYFFSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD  160
SEC-ovine      81 VVDVYGSNYYVNCCFFSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD  160
                                                                        200
                     *                     *                    ↓  .                    *  *     *      *
SEC1          161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVHLTTKNGX  240
SEC2          161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFQYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVHLTTKNGX  240
SEC3-FRI913   161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFQYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVHLTTKNGX  240
SEC3-FRI909   161 IKARNFLINKKNLYEFNSSPYETGYIKFIESNGNTFWYDMMPAPGDKFDQSKYLMIYKDNKMVDSKSVKIEVHLTTKNGX  240
SEC-MNCopeland 161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFQYDMMPAPGDKFDQSKYLMMYNDNKTVDSKRVKIEVHLTTKNGX  240
SEC-4446      161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFQYDMMPAPGDKFDQSKYLMMYNDNKTVDSKRVKIEVHLTTKNGX  240
SEC-bovine    161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFQYDMMPAPGDKFDQSKYLMMYNDNKTVDSKRVKIEVHLTTKNGX  240
SEC-ovine     161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFQYDMMPAPGDKFDQSKYLMMYNDNKTVDSKRVKIEVHLTTKNGX  240
```

Table 3A.

Conserved Regions of Enterotoxin Molecules*

Toxin	Region 1 Residue #	Region 1	Toxin	Region 3 Residue #	Region 3
SEA	79	KYKGKKVDLYG	SEA	147	KKNVTVQELDLQARRYL
SEB	76	KYKDKYVDVFG	SEB	152	KKKVTAQELDYLTRHYL
SEC1	76	KYKDEVVDVYG	SEC1	151	KKSVTAQELDIKARNFL
SEC2	76	KYKDEVVDVYG	SEC2	151	KKSVTAQELDIKARNFL
SEC3	76	KYKDEVVDVYG	SEC3	151	KKSVTAQELDIKARNFL
SED	74	HFKSKNVDVYP	SED	142	KKNVTVQELDAQARRYL
SEE	76	KYKGKKVDLYG	SEE	144	KKEVTVQELDLQARHYL
SPEA	70	LFKDKNVDIYG	SPEA	137	KKMVTAQELDYKVRKYL
SPEC	63	FKRDDHVDVFG	SPEC	124	KDIVTFQEIDFKIRKYL
TSST-1	56	FTKGEKVDLNT	TSST-1	121	KK----Q-L-I
			TSST-1	129	LDFEIRHQL

* From Hoffmann et al., *Infect Immunol* 62:3396 (1994).

Table 3B.

Conserved Regions of Enterotoxin Molecules (Cont.)*

Toxin	Region 2 Residue #		Toxin	Region 4 Residue #	
SEA	106	CMYGGVILHDNN	SEA	209	LLRIYRDNKTINSE
SEB	113	CMYGGVTEHNGN	SEB	213	YLMMYNDNKMVDSK
SEC1	110	CMYGGITKHEGN	SEC1	213	YLMMYNDNKTVDSK
SEC2	110	CMYGGITKHEGN	SEC2	213	YLMMYNDNKTVDSK
SEC3	110	CMYGGITKHEGN	SEC3	213	YLMIYKDNKMVDSK
SED	101	CTYGGVTPHEGN	SED	204	QLRIYSDNKTLSTE
SEE	103	CMYGGVTLHDNN	SEE	206	LLRIYRDNKTINSE
SPEA	98	CIYGGVTNHEGN	SPEA	197	YLMIYKDMETLDSN
SPEC	85	YIYGGITPAQNN	SPEC	184	IFAKYKDNRIINMK
TSST-1	83	FQISGVTNTEKL	TSST-1	179	PPINIDEIKTIEAE

* From Hoffmann et al., *Infect Immunol* 62:3396 (1994).

TABLE 4.

SEC1 LOOP MUTANTS

AMINO ACID #	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110
SEC1 (wild type)																		
AMINO ACID	Cys	Tyr	Phe	Ser	Ser	Lys	Asp	Asn	Val	Gly	Lys	Val	Thr	Gly	Gly	Lys	Thr	Cys
NUCLEIC ACID	TGC	TAT	TTT	TCA	TCC	AAA	GAT	AAT	GTA	GGT	AAA	GTT	ACA	GGT	GGC	AAA	ACT	TGT
SEC1 Loop Deletion Mutants							301	--	306									
−4 A.A. MUTANT	Cys	Tyr	Phe	Ser	Ser	Lys	Asp	Asn	Ala	————				Gly	Gly	Lys	Thr	Cys
	TGC	TAT	TTT	TCA	TCC	AAA	GAT	AAT	GCA					GGT	GGC	AAA	ACT	TGT
−9 A.A. MUTANT	Cys	Tyr	Phe	Ser	Ser	————									Gly	Lys	Thr	Cys
	TGC	TAT	TTT	TCA	TCC										GGC	AAA	ACT	TGT
−12 A.A MUTANT	Cys	Cys	————											GT	Gly	Lys	Thr	Cys
	TGC	T—													GGC	AAA	ACT	TGT

WHAT IS CLAIMED IS:

1. A modified pyrogenic toxin derived from a native disulfide loop–containing pyrogenic toxin, wherein the modified toxin comprises a disulfide loop region containing no more than 10 amino acid residues.

2. The modified toxin of claim 1 wherein the native disulfide loop–containing pyrogenic toxin is a staphylococcal toxin or a streptococcal toxin.

3. The modified toxin of claim 2 wherein the staphylococcal toxin is a type A, B, C, D, E, G, or H staphylococcal enterotoxin.

5. The modified toxin of claim 2 wherein the streptococcal toxin is streptococcal pyrogenic exotoxin A or streptococcal superantigen.

6. The modified toxin of claim 1 wherein the disulfide loop region contains no more than 6 amino acid residues.

7. The modified toxin of claim 1 wherein the native disulfide loop–containing pyrogenic toxin is a type C staphylococcal enterotoxin.

8. The modified toxin of claim 7 wherein the modification comprises a deletion of at least 8 amino acid residues within the disulfide loop region.

9. The modified toxin of claim 8 wherein the modification comprises a deletion of amino acid residues 98–106.

10. The modified toxin of claim 7 wherein the modification comprises deletion of at least 12 amino acid residues within the disulfide loop region.

11. The modified toxin of claim 10 wherein the modification comprises deletion of amino acid residues 95–106.

12. The modified toxin of claim 7 wherein the type C staphylococcal enterotoxin is staphylococcal enterotoxin C1.

13. The modified toxin of claim 12 comprising a cysteine residue at position 94.

14. The modified toxin of claim 7 wherein the staphylococcal enterotoxin is staphylococcal enterotoxin C1, staphylococcal enterotoxin C2, staphylococcal enterotoxin C2, staphylococcal enterotoxin C–MNCopeland, staphylococcal enterotoxin C–4446, staphylococcal enterotoxin C–bovine, staphylococcal enterotoxin C–canine or staphylococcal enterotoxin C–ovine.

15. The modified toxin of claim 1 comprising a cysteine residue at position 2 of the disulfide loop region.

16. The modified toxin of claim 1 having substantially decreased toxicity in comparison to the native toxin.

17. The modified toxin of claim 16 having an emetic response inducing activity decreased by at least about 100–fold in comparison to the native toxin.

18. The modified toxin of claim 16 having an fever inducing activity decreased by at least about 100–fold in comparison to the native toxin.

19. The modified toxin of claim 16 having an LD_{50} in Dutch Belted rabbits which is at least about 100–fold higher than the native toxin.

20. A modified pyrogenic toxin derived from a native disulfide loop–containing pyrogenic toxin, wherein the modification comprises deletion of at least about 40% of the amino acid residues within the disulfide loop of the native toxin.

21. The modified pyrogenic toxin of claim 20 comprising a cysteine residue at position 2 of the disulfide loop region.

22. The modified pyrogenic toxin of claim 20 wherein the native pyrogenic toxin is a type C staphylococcal enterotoxin.

23. An isolated nucleotide acid comprising a nucleotide sequence encoding a modified pyrogenic toxin derived from a native disulfide loop–containing pyrogenic

toxin, wherein the modified toxin comprises a disulfide loop region which includes no more than 10 amino acid residues.

24. An isolated nucleotide acid comprising a nucleotide sequence encoding a modified staphylococcal enterotoxin derived from a native staphylococcal enterotoxin, wherein the modification comprises deletion of at least about 40% of the amino acid residues within the disulfide loop region of the native enterotoxin.

25. A modified pyrogenic toxin derived from a native type C staphylococcal enterotoxin, wherein the modified toxin comprises a disulfide loop region which includes no more than 10 amino acid residues.

26. The modified toxin of claim 25 wherein the disulfide loop region is Cys–Cys–Gly–Lys–Thr–Cys.

Attorney Docket No. 12136.1USWO

MERCHANT & GOULD P.C.

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: NON-TOXIC IMMUNE STIMULATING ENTEROTOXIN COMPOSITIONS

The specification of which
a. ☐ is attached hereto
b. ☒ was filed on 24 May 2000 as application serial no. and was amended on 24 May 2000 (if applicable) (in the case of a PCT-filed application) described and claimed in international no. PCT/US98/25107 filed 01 December 1998 and as amended on (if any), which I have reviewed and for which I solicit a United States patent.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56 (attached hereto).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on the basis of which priority is claimed:

a. ☒ no such applications have been filed.
b. ☐ such applications have been filed as follows:

FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC § 119			
COUNTRY	**APPLICATION NUMBER**	**DATE OF FILING (day, month, year)**	**DATE OF ISSUE (day, month, year)**
ALL FOREIGN APPLICATION(S), IF ANY, FILED BEFORE THE PRIORITY APPLICATION(S)			
COUNTRY	**APPLICATION NUMBER**	**DATE OF FILING (day, month, year)**	**DATE OF ISSUE (day, month, year)**

I hereby claim the benefit under Title 35, United States Code, § 120/365 of any United States and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. APPLICATION NUMBER	**DATE OF FILING (day, month, year)**	**STATUS (patented, pending, abandoned)**
PCT/US98/25107	01 December 1998	Inactive

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

U.S. PROVISIONAL APPLICATION NUMBER	**DATE OF FILING** (Day, Month, Year)
60/067,357	02 December 1997

I hereby appoint the following attorney(s) and/or patent agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

Albrecht, John W.	Reg. No. 40,481
Anderson, Gregg I.	Reg. No. 28,828
Batzli, Brian H.	Reg. No. 32,960
Beard, John L.	Reg. No. 27,612
Berns, John M.	Reg. No. 43,496
Black, Bruce E.	Reg. No. 41,622
Branch, John W.	Reg. No. 41,633
Bremer, Dennis C.	Reg. No. 40,528
Bruess, Steven C.	Reg. No. 34,130
Byrne, Linda M.	Reg. No. 32,404
Carlson, Alan G.	Reg. No. 25,959
Caspers, Philip P.	Reg. No. 33,227
Chiapetta, James R.	Reg. No. 39,634
Clifford, John A.	Reg. No. 30,247
Cochran, William W.	Reg. No. 26,652
Daignault, Ronald A.	Reg. No. 25,968
Daley, Dennis R.	Reg. No. 34,994
Dalglish, Leslie E.	Reg. No. 40,579
Daulton, Julie R.	Reg. No. 36,414
DeVries Smith, Katherine M.	Reg. No. 42,157
DiPietro, Mark J.	Reg. No. 28,707
Edell, Robert T.	Reg. No. 20,187
Epp Ryan, Sandra	Reg. No. 39,667
Glance, Robert J.	Reg. No. 40,620
Goggin, Matthew J.	Reg. No. 44,125
Golla, Charles E.	Reg. No. 26,896
Gorman, Alan G.	Reg. No. 38,472
Gould, John D.	Reg. No. 18,223
Gregson, Richard	Reg. No. 41,804
Gresens, John J.	Reg. No. 33,112
Hamre, Curtis B.	Reg. No. 29,165
Hillson, Randall A.	Reg. No. 31,838
Holzer, Jr., Richard J.	Reg. No. 42,668
Johnston, Scott W.	Reg. No. 39,721
Kadievitch, Natalie D.	Reg. No. 34,196
Karjeker, Shaukat	Reg. No. 34,049
Kastelic, Joseph M.	Reg. No. 37,160
Kettelberger, Denise	Reg. No. 33,924
Keys, Jeramie J.	Reg. No. 42,724
Knearl, Homer L.	Reg. No. 21,197
Kowalchyk, Alan W.	Reg. No. 31,535
Kowalchyk, Katherine M.	Reg. No. 36,848
Lacy, Paul E.	Reg. No. 38,946
Larson, James A.	Reg. No. 40,443
Liepa, Mara E.	Reg. No. 40,066
Lindquist, Timothy A.	Reg. No. 40,701
Lycke, Lawrence E.	Reg. No. 38,540
McAuley, Steven A.	Reg. No. 46,084
McDonald, Daniel W.	Reg. No. 32,044
McIntyre, Jr., William F.	Reg. No. P-44,921
Mueller, Douglas P.	Reg. No. 30,300
Pauly, Daniel M.	Reg. No. 40,123
Phillips, John B.	Reg. No. 37,206
Plunkett, Theodore	Reg. No. 37,209
Prendergast, Paul	Reg. No. 46,068
Pytel, Melissa J.	Reg. No. 41,512
Qualey, Terry	Reg. No. 25,148
Reich, John C.	Reg. No. 37,703
Reiland, Earl D.	Reg. No. 25,767
Schmaltz, David G.	Reg. No. 39,828
Schuman, Mark D.	Reg. No. 31,197
Schumann, Michael D.	Reg. No. 30,422
Scull, Timothy B.	Reg. No. 42,137
Sebald, Gregory A.	Reg. No. 33,280
Skoog, Mark T.	Reg. No. 40,178
Spellman, Steven J.	Reg. No. 45,124
Stoll-DeBell, Kirstin L.	Reg. No. 43,164
Sumner, John P.	Reg. No. 29,114
Swenson, Erik G.	Reg. No. 45,147
Tellekson, David K.	Reg. No. 32,314
Trembath, Jon R.	Reg. No. 38,344
Underhill, Albert L.	Reg. No. 27,403
Vandenburgh, J. Derek	Reg. No. 32,179
Wahl, John R.	Reg. No. 33,044
Weaver, Karrie G.	Reg. No. 43,245
Welter, Paul A.	Reg. No. 20,890
Whipps, Brian	Reg. No. 43,261
Wickhem, J. Scot	Reg. No. 41,376
Williams, Douglas J.	Reg. No. 27,054
Witt, Jonelle	Reg. No. 41,980
Wu, Tong	Reg. No. 43,361
Xu, Min S.	Reg. No. 39,536
Zeuli, Anthony R.	Reg. No. 45,255

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Merchant & Gould P.C. to the contrary.

Please direct all correspondence in this case to Merchant & Gould P.C. at the address indicated below:

Merchant & Gould P.C.
P.O. Box 2903
Minneapolis, MN 55402-0903

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2	**Full Name Of Inventor**	**Family Name** BOHACH	**First Given Name** Gregory	**Second Given Name** L.
0	**Residence & Citizenship**	**City** Moscow	**State or Foreign Country** Idaho	**Country of Citizenship** U.S.A
1	**Post Office Address**	**Post Office Address** 300 Rose Court	**City** Moscow	**State & Zip Code/Country** Idaho 83844 / U.S.A.
Signature of Inventor 201:			**Date:** 6/22/00	